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EXTRACELLULAR DNA IN THE BLOOD OF PREGNANT WOMEN

V. I. Kazakov, V. M. Bozhkov, V. A. Linde, M. A. Repina, V. M. Mikhaylov

Institute of Cytology, Russian Academy of Sciences, and Medical Academy of Postgraduate Physician Education, St. Peterburg

The level of extracellular DNA increases in the blood of women during pregnancy. By means of PCR, the full-size Alu repeats were observed among extracellular blood DNA repeats of pregnant women. Furthermore, with Tc65 type primer the PCR method allowed to observe in the blood DNA fragments flanked by inverted Alu repeats (inter Alu repeats). The presence of such a type of inter Alu repeats was estimated in the blood of women being in the first trimester of pregnancy only, but was not estimated among blood DNA fragments of women of the last trimester of pregnancy. It is discussed which types of cells may serve as a source of extracellular blood DNA (either trophoblast cells, lymphocytes, or decidual cells), the significance of such DNA for pregnancy being appreciated.

It has been shown that extracellular DNA is contained in the blood of humans and animals (Stroun et al., 1977; Fedorov, Yaneva, 1982; Vladimirov et al., 1992). An increase in the content of extracellular DNA in the blood of humans has been described during pathological processes taking place in various types of tissue of the body, especially during certain inflammatory processes of the gastrointestinal tract, during tumor and infectious diseases of viral etiology, and during disseminated lupus (Anker et al., 1975; Leon et al., 1977; Shapiro et al., 1983; Stroun et al., 1987; Goto et al., 1991).

The molecular mass of the extracellular DNA of the blood is on average between $1 \cdot 10^6$ to $15 \cdot 10^6$ Da (Stroun et al., 1977; Fedorov, Yaneva, 1982). According to EM microscopy data, the extracellular DNA of healthy persons is double-strand and linear (Dennin, 1979). According to Vasyukhin et al. (1991), human extracellular DNA contains unique sequences. It has also been shown that the extracellular DNA of the blood during disseminated lupus has sequences capable of forming Z-DNA (Van Helder, 1985).

It is believed that the high-molecular component of extracellular DNA in the blood comes from living cells (Stroun et al., 1977). It has been demonstrated in vitro that certain types of cells, especially lymphocytes, excrete extracellular DNA into their surroundings (Rogers et al., 1972; Rogers, 1976; Stroun et al., 1977; Fedorov, Yaneva, 1982). Furthermore, the blood also contains low-molecular DNA, corresponding to nucleosomes in its mobility. In the blood of rats, its content increases after total X-ray exposure (Belokhvostov et al., 1987; Vladimirov et al., 1992; Tishchenko et al., 1993). According to Tishchenko et al. (1993), the low-molecular DNA in the blood of rats after total irradiation is enriched in GC sequences. It has been conjectured that the low-molecular DNA in the blood is a product of intensified extrachromosomal synthesis

of ring DNA, detected in many types of cells of humans and mammals (Vladimirova et al., 1992). Our attention was drawn to the proposition that the rise in the level of extracellular low-molecular DNA in the blood of rats after irradiation is the consequence of increased activity of Ca/Mg-endonuclease in the cells (Tishchenko et al., 1993). This proposition is in good agreement with data on the intensification of the process of apoptosis in the cells of various tissues of the body after irradiation (Khanson, Komar, 1985).

It is clear from the above that analysis of the extracellular DNA in the blood of humans and animals is of both theoretical and practical interest. It is not ruled out that the nucleotide composition of the extracellular DNA of blood is not so random as not to reflect the peculiarities of the processes of differentiation and cell death taking place in various tissues at each particular moment in the life of the organism. All of this dictated our choice of the blood of pregnant women as the object of our research. According to available data, cellular proliferation, differentiation, and cell death occur in the uterus during pregnancy (Fedorova, Kalashnikova, 1986; Mikhaylov et al., 1989, 1992a, 1992b; Mikhaylov, 1993). It was anticipated that these processes exert an influence on the specifics of the nucleotide composition of the extracellular DNA in the blood of pregnant women.

Material and method

We studied the blood sera of men, nonpregnant women, and women in the first and third trimester of pregnancy and those with late toxicosis of pregnancy. The blood was taken by syringe from the cubital vein under sterile conditions, placed in a centrifuge test tube, and left at room temperature until clotted. Immediately after the formation of a thrombus, it was removed from the walls of the test tube and centrifuged at 400 g for 10 min. The serum was centrifuged yet again at 2000 g for 10 min at 4º C. The serum obtained in this way was kept at -60º C. The use of serum instead of plasma for the analysis of the DNA of the blood can be justified if one observes the conditions for formation of a thrombus at room temperature and immediate removal of the serum from the thrombus (Leon et al., 1977; Shapiro et al., 1983). Such was done in the present investigation.

After this the serum was treated twice with phenol, mixtures of phenol and chloroform (1:1), chloroform, and isoamyl alcohol (24:1) and precipitated with ethanol at -20º C. The DNA preparations were analyzed in 1% agarose gel, and then used as the matrix in a polymerase chain reaction (PCR).

The PCR was carried out using thermophilic DNA polymerase from Thermus thermophilus. We performed 30-35 amplification cycles. The amplified fragments were analyzed in 8% PAAG. The annealing temperature of the primers was 55º C for primers B1 and C2 and 60º C for primer Tc65, the concentration of Mg^{2+} ions being 2-5 mM. The primers for the PCR were synthesized by us with the solid-phase triether phosphite method in the ß-cyanoethyl modification on the Gene assembler instrument from Pharmacia. The sequence of the Tc65 primer was taken from literature sources (Nelson et al., 1989), the sequences of the other primers were found with the help of the Oligo program (Microsoft) for the consensus sequence of Alu repeats of the MF family and designated as B1 and C2 by us.

When the PCR was carried out with the pair of primers B1 and C2, an Alu repeat with a length of 239 base pairs was amplified as a result of the reaction. When only one primer Tc65 was used in the PCR, DNA fragments were amplified that were flanked by two Alu repeats with their terminal 3’ regions facing each other (inter-Alu repeats).

The sequences of the primers are:

Tc65—5′—AAGTCGCGGCCGCTTGCAGTGAGCCGAGA—3′
B1—5′—CCTGTAATCCCAGCACTTTGGGAGGC—3′
C2—5′—CCCAGGC$^{T}_{G}$GGAGTGC$^{A}_{G}$GTGG—3′

Results and discussion

It was not our goal to study in detail the changes in the concentrations of DNA in the blood serum of pregnant women. From the data of authors using radioimmunological methods of determination (Leon et al., 1977; Shapiro et al., 1983), the concentration of DNA in the blood sera of healthy donors varied from 0 to 0.1 mcg/ml; according to authors using biochemical methods (Anker et al., 1975; Stroun et al., 1987), from 0.1 to 0.6 mcg/ml. Our data on the

concentration of DNA in the blood of men and nonpregnant women corresponded to the literature data.

According to our data findings, during pregnancy there is an increase first of all in the concentration of low-molecular DNA, the increase being most pronounced during gestosis. The size of such DNA is from 150 to 2500 base pairs. There is a belief that the increase in concentration of low-molecular extracellular DNA in the blood is a consequence of increased synthesis of extrachromosomal DNA (Vladimirov et al., 1992). However, in evaluating the data presented, one must consider that a substantial number of endometrial cells perish during pregnancy, especially at the end of pregnancy and in connection with gestosis (Kottsova et al., 1989; Mikhaylov et al., 1992a). According to our data, the death of decidual cells is attended by a gradual loss of nuclear DNA and fragmentation of peripheral parts of the nuclei and cytoplasm and, apparently, this occurs by apoptosis (Mikhaylov et al., 1992a, 1992b; Mikhaylov, 1993). One of the major mechanisms of cell death of the apoptosis type is internucleosomal degradation of DNA due to activation of Ca^{2+} endonuclease (Wyllie, 1980). This does not rule out the possibility of extrachromosomal copying of DNA as the source of the extracellular DNA in the blood. Regardless of whether intensified synthesis of extrachromosomal DNA or apoptotic cell death is the source of the extracellular DNA in the blood, the presence of Alu repeats in it testifies to its nuclear origin (Fig. 1; see insert VIII).

Worthy of attention is the presence of inter-Alu repeats among the DNA of the blood serum of pregnant women. It is known that approximately a third of the Alu repeats of humans belongs to the inverted repeats, and the mean distance between inverted pairs is approximately 50 t.p.n. (Jelinek, Schmid, 1982). The bulk of such inverted repeats apparently contain a unique internal fragment. There are several kinds of inter-Alu repeats. With the help of the Tc65 primer, we detected inter-Alu repeats in the blood of women only in the first trimester of pregnancy (Fig. 2). When the PCR was carried out with the Tc65 primer, only those DNA fragments are amplified in which the spacer DNA is flanked by Alu repeats mutually facing each other by their 3’ regions, which makes these fragments even more unique. Thus, if the presence in the blood of pregnant women of an increased quantity of low-molecular DNA fragments is a sign of increased cell death occurring in the uterus in all phases of pregnancy, then the presence of inter-Alu repeats indicates the functioning of other mechanisms for such sequences getting into the serum. It is important that inter-Alu repeats have been detected only in the blood of women in the first trimester of pregnancy. This fact most likely reflects the difference in content of the cellular processes that are characteristic of the early and late stages of pregnancy. While at the end of pregnancy there is observed a death of decidual cells, which explains increase in concentration of low-molecular DNA in the blood; in the initial period of pregnancy when implantation and placentation of the embryo in the uterus occurs, there is decidualization of the endometrium and growth of the embryonal vesicle, i.e., a process of differentiation of the cells of the uterine mucous membrane and cells of the trophoblast.

It is likely that, in the early stages of pregnancy, an excretion of inter-Alu repeats from the cells occurs. Which type of cells can excrete inter-Alu repeats is a matter of great interest. This question also confronts investigators when discussing the sources of high-molecular serum DNA (see the survey: Fedorov, Yaneva, 1982). Excretion of DNA in vitro has been well characterized for lymphocytes (Fedorov, Yaneva, 1982). It is assumed that the lymphocytes might be the source of such DNA during tumor diseases. In the early stages of pregnancy, substantial lymphocyte infiltration is also observed in the uterine mucous membrane.

Thus, in the early stages of pregnancy in humans, cells of the fetus (trophoblasts) and the mother (cells of the endometrium and lymphocytes) may excrete DNA. In view of the above, and also considering the transposonic and recombinogenic nature of the Alu repeats (Tomilin, 1992), it can be conjectured that the inter-Alu repeats discovered by us in the blood serum of pregnant women may play some kind of regulatory role in the early stages of pregnancy. The cloning and sequencing of these fragments is of particular interest. What has been said does not rule out the presence of other inter-Alu repeats in the blood of pregnant women, which can be identified by means of other primers and may have their own features of distribution in the blood in the course of pregnancy.

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EXTRACELLULAR DNA OF PREGNANT WOMEN BLOOD

V. I. Kazakov, V. M. Bozhkov, V. A. Linde, M. A. Repina, V. M. Mikhailov

Institute of Cytology, Russian Academy of Sciences, and Medical Academy of Postgraduate Education, St. Petersburg

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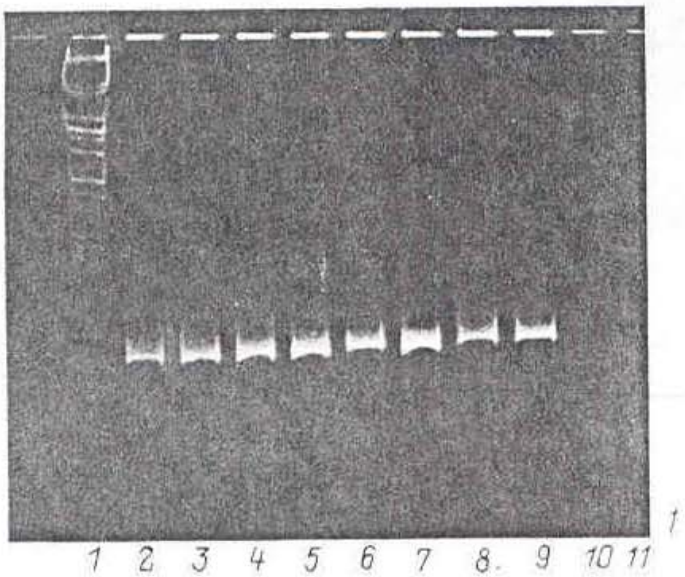

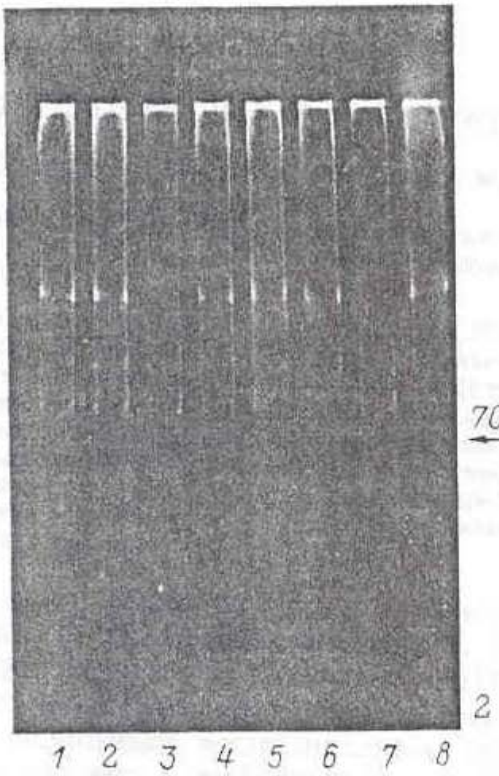

Рис. 1. Фрагменты ДНК, полученные в результате проведения полимеразной цепной реакции с парой праймеров B1 и C2. Синтез полноразмерного Alu-повтора.

1 — ДНК фага λ, рестрицированная по PstI; *2* — ДНК из сыворотки крови мужчин; *3* — ДНК из сыворотки крови небеременной женщины; *4* — ДНК из сыворотки крови женщины I триместра беременности; *5* — ДНК из сыворотки крови женщины III триместра беременности; *6* — ДНК из сыворотки крови при гестозе; *7* — ДНК из тромба крови женщин III триместра беременности; *8* — тотальная ДНК человека; *9* — плазмида PaII с клонированным Alu-повтором; *10* — реакция без матрицы; *11* — реакция без праймера.

Рис. 2. Фрагменты ДНК, полученные в результате проведения полимеразной цепной реакции с праймером Tc65 (интер-Alu-повтор).

1—8 — ДНК из сыворотки крови различных женщин I триместра беременности.